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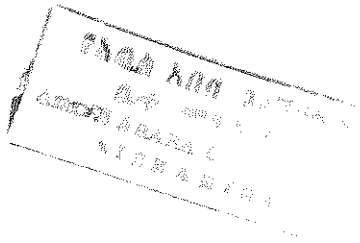
In vitro T-Cell Responses as Surrogate Markers for HIV-1Infection Progression in Ethiopia



By

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A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial fulfilment of the requirement for the degree of Master of Science in Immunology

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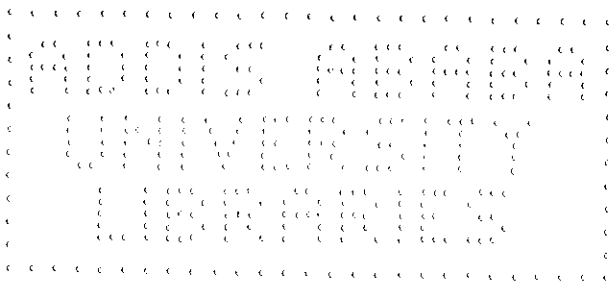

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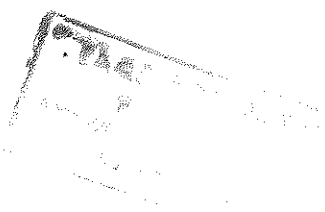
List of Abbreviations

AAC - Activation Associated Necrosis
AIDS- Acquired Immunodeficiency Syndrome
ARV - AIDS Associated Retrovirus
APC - Antigen Presenting Cell
Ag - Antigen
Ab - Antibody
BCG - Bacille Calmette Guerin
BrdU - 5-Bromo- 2-deoxyuridine
CCR - Chemokine Receptor
CD - Cluster of Differentiation
CR -Complement Receptor
CSW - Commercial Sex Worker
CTL - Cytotoxic T Lymphocyte
DC - Dendritic Cell
DMSO - Dimethyl Sulphoxide
DTH delayed-type hypersensitivity
EBSS - Earel's Balanced Salt Solution
ECM -Extracellular Matrix
EDTA - Ethylene Diamine Tetra acitic Acid
ELISA -Enzyme Linked Immunosorbent Assay
ENARP -Ethiopian-Netherlands AIDS Research Project
Env - Envelope
FACS - Fluoresence Activated Cell Sorter
Fas CD95 antigen
FCS - Fetal Calf Serum
FDC - Follicular Dendritic Cell
Gag - Group specific antigen
GP - Glycoprotein
GuSCN Guanidinium Isothiocynate



HLA - Human Leukocyte Antigen

HIV - Hunan Immunodeficiency Virus

HIV+ - HIV- seropositive

HIV- - HIV-seronegative

HTLV-III Human T-cell Lymphotropic Virus type III

HRP - Horse Radish Peroxidase

IFN - Interferon

Ig - Immunoglobulin

IL - Interleukin

IMDM - Iscove's Modfied Dulbecco's Medium

LAV - Lymphadenopathy Associated Virus

LFA - Leukocyte Function Antigen

LN - Lymph Node

LTR -Long Terminal Repeat

mAb -monoclonal Antibody

MHC -Major Histocompatibility Complex

MIP Macrophage Inflammatory Protein

M-tropic - Macrophage tropic

NASBA - Nucleic Acid Sequence Based co-Amplification Assay

NBCS -New Born Calf Serum

NC -Negative Control

Nef -Negative regulatory f actor

NF-KB - Nuclear Factor kB

NK -Natural Killer

NSI - Non-Syncytium Inducing

OD - Optical Density

PBMC - Peripheral Mononuclear Cells

PC - Positive Control

PCR Polymerase chain reaction

PHA - Phytoheamagglutinin

PPD - Purified Protein Derivative

RANTES-	Regulation on Activation Normal T cell Expressed and Secreted
RT	Room Temperature
SD	standard deviation
SI	Syncytium Inducing
STD	Sexual Transmitted Disease
Tat	trans-activator gene
TB	Tuberculosisa
TCR	T Cell Receptor
TD	T cell Dependent
TH	T Hellper
TMB	Tetramethyl benzidine
TNF	Tumour necrosis factor
Vif	Virion infectivity factor
VPU	Viral protein U
WHO	World Health Organization

ABSTRACT

In an effort to use immunological abnormalities as markers of HIV-1 infection progression in Ethiopian individuals, this work assessed the changes in *in vitro* and *in vivo* T-cell responses to recall *M. tuberculosis* antigen, purified protein derivative (PPD). This was further evaluated against CD4+ and CD8+ T-cells count and viral load in the same subjects. Peripheral blood mononuclear cells (PBMCs) isolated from 36 HIV-1 seropositive and 105 seronegative individuals were subjected to 6 days *in vitro* PPD stimulation and cytokine production. The T-cell proliferative response was then evaluated using 5-bromo-2-deoxyuridine (BrdU) instead of thymidine incorporation as a method of assay. Cytokines (IFN-γ and IL-4) production was measured in the PBMC culture supernatants using sandwich ELISA. Three days phytoheamagglutinin (PHA) stimulation responses were used as positive controls. *In vivo* tuberculin PPD responses were measured for 18 HIV-1 seropositive and 74 seronegative individuals by standard Mantoux reaction. Results show that T-cell responses to PPD both *in vitro* and *in vivo* were affected by HIV-1 infection, in contrast to HIV-1 seronegatives. Significant reduction in IFN-γ production was also observed between the two groups following PPD stimulation ($p = 0.000$), but not with IL-4. Thus, a shift from Th1 to Th2 cytokines production was not observed. Analysis of T–cell responses to PPD showed a positive correlation with CD4+ T cell counts, and negative with plasma viral load. This confirmed that HIV-1 infection progression is followed by a depletion of CD4+ T cells count and high viral load. Change in proliferation and cytokine production was specific to PPD. It suggests that *Mycobacterium tuberculosis* specific immune responses are affected in HIV-1 infected subjects. Taken together our results indicated that evaluating T-cell responses to recall antigen, PPD can be used as an early marker for HIV-1 infection progression.

of unemployment, sexual promiscuity and the presence of a wide diversity of HIV-1 subtypes in Africa have also been indicated as powerful components in HIV-1 spread and transmission.

The virus has profound economic and social implications for both developed and developing countries. No country in the world is free of the disease and can claim that it has stopped its spread. The most effective and efficient method to prevent HIV-infection and disease manifestation would be through vaccination and effective treatment, which currently are not available. The generally accepted method of implementing preventive strategies for HIV infection is education that will inform and increase awareness of the society.

1.1 Overview of HIV infection in Ethiopia

The first HIV positive sera in Ethiopia were reported in 1984, and the first AIDS cases in 1986 (Eshete and Sahlu, 1996). Since then the epidemic has spread fast, the estimated number of AIDS cases and HIV infected individuals in Ethiopia by the end 1991 were about 14000 and 300,000 respectively, and the country ranked 13th in Africa in the total number of reported AIDS cases in 1992 (Ayehunie, 1992). Although HIV infection has been reported from all provinces of the country with different magnitudes (Sahlu, 1999), the epidemic seems to be a rapidly growing problem initially among commercial sex works (CSWs) in Addis Ababa, the capital city of the country and truck drivers for the last ten years (Mehret *et al.*, 1990, Eshete and Sahlu *et al.*, 1996, Fontanet *et al.*, 1998). A rapid spread of HIV infection has been observed in the country within a few years compared to other central and East African countries with a high prevalence in the 25-29 year age group. The estimated number of HIV-infected individuals by the end of 1999 was 3000,000, and it has been suggested that the country has the third largest HIV-infected population in the world today (WHO., 1999, Sahlu *et al.*, 1999). study has indicated that the predominant presence of HIV-1 subtype C in Ethiopia (Abebe et al., 1997). Sub-type C has become the prevalent subtype in the world. According to a report of (WHO, 1999) of all the HIV-1 infected people in the world, 56% carry the C subtype

with the predominant in countries like South Africa and Ethiopia.

Although other possible modes of transmission of the virus cannot be excluded, heterosexual transmission is the major route of HIV spread in Ethiopia (Mehret *et al.*, 1990, Ayehunie, 1992). Therefore, risk factors like previous episodes of sexually transmitted diseases (STDs), frequent change of sexual partners, and unprotected sexual practice are considered to be the main contributors for the rapid spread of the virus in the country (Mehret *et al.*, 1990, Taffa, 1998, Sahlu *et al.*; 1999).

1.2 The Virus

HIV is a retrovirus belonging to the subfamily of lentiviruses in the family of retroviridae. Like other members of the retroviral family, the HIV-1 virion consists of an outer membrane, formed by a lipid bilayer derived from the host cell membrane. Its provirus contains two long terminal repeats (LTR) at each end, along with three structural genes (gag, pol and env) that are essential for virus replication. The gag gene encodes a polyprotein precursor that is subsequently cleaved by the viral protease during maturation, whereas the pol gene encodes a precursor protein for the viral enzymes (reverse transcriptase, integrase and protease) and the env gene encodes a glycosylated polypeptide precursor glycoprotein 160 (gp160) that is processed to form the exterior glycoportein (gp120) and the transmembrane glycoprotein (gp41). In addition to these genetic elements, HIV contains at least six accessory genes: tat, rev, nef, vif, vpu and vpr (HIV-1) or vpx (HIV-2) which have regulatory functions in the life cycle of the viruses (Haseltine, 1988, Fauci and Lane, 1998) (Fig.1).

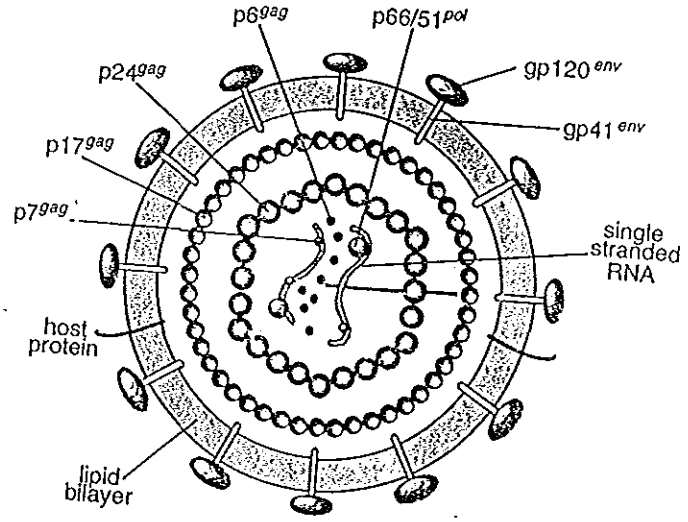


Figure 1. Schemtic representation of an HIV virion.

HIV infects white blood cells, primarily the CD4+ cells (Fauci, 1988, Wahl *et al.*, 1999). The entry of the virus into the host cell is mediated by interactions between the virus envelope glycoproteins, gp120 and gp41, and the surface molecule receptors of the CD4+ cells, CD4 and members of the chemokine receptor family mainly CCR5 and CXCR4 (Kwong *et al.*, 1998).

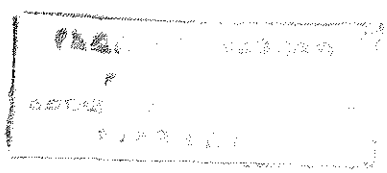
After it enters into the body, antigen-presenting cells (APCs) such as follicular dendritic cells (FDCs), blood dendritic cells as well as mucosal dendritic cells transport or present the virus to the susceptible target cells (CD4+ T cells) (Mosier and Sieburg, 1994). Following the binding of gp120 to CD4 and co-receptors, the virus is internalized, uncoated, and inserts a DNA copy of its viral RNA into the genome of the host cell as provirus (Fauci, 1988). This initial infection with HIV is followed by a long and variable

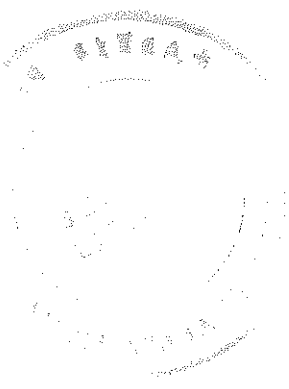
asymptomatic period that is characterized by viral latency or low level of virus replication (Rosenberg and Fauci, 1990, Fox and Cottler-Fox, 1992). Occasionally the virus may get activated, because of other infections activating the immune system of the host. For example, infections with *Mycobacterium tuberculosis* or parasites can result in temporary increases in viral loads and decline in CD4+ T cell numbers feature most common in African HIV-infected individuals (Bentwich, *et al.*, 1995, Goletti *et al.*; 1996 Kalinkovich *et al.*, 1998).

1.3 Immune Alterations Following HIV Infection

The immune system is a complex system that consists of two main components: the innate and the adaptive. It protects the host against invasive organisms such as bacteria, viruses, fungi and parasites, in addition to functioning as a surveillance network to guard against the growth and dissemination of tumor cells. The first line of defense is provided by cells and molecules of the innate immunity which includes phagocytic cells, natural killer (NK) cells, mast cells and eosinophils, as well as complement components and pro-inflammatory cytokines. When this is overcome, the specific or adaptive part of the immune system which consists of two classes of lymphocytes (T and B cells) are involved. B cells produce antibodies under the help of T cells, which are able to protect from microbes living outside cells. On the other hand, the T cells mediate helper/inducer, suppressor and cytotoxic activities (Romagnani, 1997).

However, infection by HIV is known for debilitating the capacity of the immune system and beginning at the time of seroconversion (Fox and Cottler-Fox, 1992). Although all components of the immune system can be altered following HIV infection (Miedema *et al.*, 1988, Fauci, 1988), the most frequently reported immunological abnormalities include changes in quantitative T lymphocytes, reduced T lymphocytes functions, impaired activities of APCs, diminished NK activity and altered immunoglobulin production (Fauci, 1988, Meyard *et al.*; 1993, Roos *et al.*; 1996, Cao *et al.*, 1996).





1. 3.1 Monocyte/Macrophage Abnormalities

Monocyte/macrophages develop from immature hematopoietic progenitor cells in the bone marrow. They are found in many tissues in the body like in the peripheral blood compartment, in the lymph nodes and the spleen, where they serve a variety of immunoregulatory, phagocytic and secretory functions. During the immune response, these cells localize in peripheral tissues by attaching to the vascular endothelium via interactions involving the adhesion molecules leukocyte function antigen-1 (LFA-1), LFA-2 and LFA-3 expressed on the monocyte surface. Then, the monocytes traverse the endothelial barrier by producing the extracellular matrix (ECM) degrading metalloprotenases to allow passage through the vascular basement membrane (Dhawan *et al.*, 1995). Macrophages ingest microbes and other foreign matter and destroy. In addition to the antimicrobial activity, macrophages serve a number of important effector functions including antitumor activity, immuno-regulatory function, and removal of autologous cells (Broide, 1991).

Macrophages play a critical role in immune regulation through antigen presentation for T helper (TH) cells by ingesting and degrading foreign particles into peptides that can bind to MHC (major histocompatibility complex) class II molecules so that it can be recognized by the T-cell receptor (TCR). Moreover, monocyte/macrophages participate in costimulatory pathway expressing costimulatory molecules and producing accessory cytokines such as interlekuine-1 (IL-1) IL-12 and IL-10 that permit antigen recognition and proliferation of Th cells (Polyak *et al.*, 1997).

Monocyte/macrophages express the CD4 and C-C-chemokine receptors for HIV-1 isolates commonly known as macrophage tropic (M-tropic) viruses (Malykh *et al.*, 1995). Thus, monocyte/macrophages are considered as the major targets of HIV-infection and may also serve as a reservoir for the virus (Meltzer *et al.*, 1990, Horuk , 1999).

In contrast to the rapid cytopathic manifestations of HIV-1 replication in T cells,

macrophages appear less susceptible to virus-induced cytopathicity (Meltzer *et al.*, 1990, Fauci and Lane, 1998). However, a number of monocyte/ macrophage defects have been reported which may contribute to the dysregulation of the immune response seen during HIV-1 infection and disease progression. For instance, it has been well documented that peripheral blood monocytes taken from patients with AIDS exhibit both immunologic and functional abnormalities including diminished chemotaxis, impaired Fc receptor (FcR)-dependent phagocytosis, loss of class II antigen (Ag) expression and impaired class II-Ag complexe formation, decreased expression of both B7.1 and B7.2 isoforms, decreased intracellular killing activity of microorganisms, decreased ability to induce T cell stimulation and aberrant cytokine production with loss of IL-1 and IL-12 production and induction of IL-10 which is thought to play an important role in T cell dysfunction and cytokine imbalance observed in HIV-infection (Yoo *et al.*, 1996, Polyak *et al.*; 1997, Kumar *et al.*, 1999).

1.3.2 Dendritic cells

Dendritic cells (DCs) are distinct lineages that arise from CD34+ cell progenitors in the bone marrow. DCs are known as professional antigen presenting leukocytes, a feature that may be related to their expression of high levels of MHC class I and II molecules, possession of co-stimulatory molecules CD40, CD80, and CD86 and production of cytokines such as IL-12, IL-6 and interferon-gamma (IFN-γ). DCs are widely distributed throughout the body in the epithelia, blood, lymph nodes (LN), lymphoid and non-lymphoid parenchymal tissues (Steinman, *et al.*, 1991). They are called Langerhans cells (in the skin), interdigitating cells in the LNs, veiled cells (in the afferent lymphatic channels), interstitial DCs (in the heart, lungs and intestine) (Ludewig *et al.*, 1995, Tsunetsugu-Yokota *et al.*, 1995).

DCs play an essential role in the stimulation of naïve as well as memory T lymphocytes. DCs recognize, and bind foreign Ags in the tissues and process it to a form that can be recognized by T cells, and transport them via the afferent lymphatic system to the lymphoid tissues, where the Ags initiate T cell responses (Knight, 1996).

DCs express CD4 surface molecules on their membrane, therefore, they are susceptible to HIV infection (O'Doherty *et al.*, 1993). Moreover, Spira *et al* (1996) and Patterson *et al* (1998) have reported that the presence of DCs at the sites of HIV-infection such as skin, blood, and genital mucosa make them the first cells to be infected by HIV following exposure to the virus.

In addition to transmitting and spreading HIV-infection to CD4+ T lymphocytes, it has been reported that DCs from HIV-1 infected individuals have impaired capacity to stimulate T cell proliferation (Blauvelt *et al.*, 1995), decreased in number and function (Macatonia *et al.*, 1990, Patterson *et al.*, 1998). In contrast, Kalter *et al* (1991) has reported that DCs obtained from both early and late stages of HIV-infected subjects have equal ability of maintaining their Ag-presenting function.

1.3.3 Natural Killer Cells

NK cells, like all other lympho-hemopoietic cells are derived from bone marrow. NK cells are large granular lymphocytes that do not express on their surfaces the CD3 antigen or any of the known TCR (α , β , or δ, γ) but that do express CD16 and CD56 surface antigens. NK cells play important role in the early response of the immune system to many viral infections without prior sensitization. NK cells lyse a variety of target cells, such as virally infected cells, antibody coated cells, and cells from a number of different tumors through non-MHC-restricted mechanisms (Yu *et al.*, 1992).

Despite the number of circulating NK cells almost remaining constant in HIV patients, functional abnormalities in these cells have been observed throughout the course of HIV disease and the severity of these abnormalities increase as infection progresses (Bowen *et al.*, 1985, Scott-Algara *et al.*, 1992). This reduction of NK cell activity might therefore, contribute to the opportunistic infections and neoplasm, which occur during the course of HIV infection progression (Forthal *et al.*, 1997).

1.3.4 B Cell Abnormalities

In humans and other mammalian species, B lymphocytes are characterized by the presence of readily detectable surface immunoglobulins. They are the precursors of the antibody (Ab) secreting plasma cells. Antibodies are specialized proteins that perform different activities in the body, such as toxin neutralization, blocking of the attachment of virus to target cells, opsonization of bacteria, activation of complement cascade and antibody-dependent cellular cytotoxicity killing of tumor and other infectious agents (Goodman, 1991).

Viral infections that are medically important are crucially controlled by B cells (Bachmann and Zinkernagel, 1997). Viruses contain proteins that induce T cell dependent (TD) B cell responses. This TD activation step of B cells occurs primarily between B cells and already primed TH cells. When primed TH cells recognize peptide on MHC class II molecules presented on B cells, the co-stimulatory molecules B7.2 followed by B7.1 are up-regulated (Janeway *et al.*, 1992). The ensuing B7-CD28 interaction further activates TH cells and leads to expression of the CD40 ligand (CD40L) (Linsley and Ledbetter, 1993). The CD40-CD40L interaction and polar secretion of cytokines, induce B cell proliferation, Ab production and isotype switching (Armitage *et al.*, 1992).

It has been demonstrated *in vitro* that less percentage of circulating B cells express the CD4 receptor surface molecule as well as CCR_5 and CXCR4, the two recently described co-receptors for HIV-1 entry (Fritsch *et al.*, 1998, Forster *et al.*; 1998, Hori *et al.*,1998).In addition to these main receptors, HIV-1 can enter into B cells by means of complement receptors such as complement receptor 1 (CR1) and CR2 (Gras *et al.*, 1993, Legendre *et al.*, 1996). Therefore, HIV-1 can infect and replicate in B cells. It is also well documented that HIV-1 infection causes functional defects in B lymphocytes although a reduction in these cells has not been well described.

A profound disturbance in B cell function is a distinctive feature of virtually all HIV-infected subjects and is most evidenced by unusually high immunoglobulin (Ig) levels

(Shirai *et al.*, 1992), high level of circulating auto-antibodies and immune complexes, hyper-IgE syndromes, (Amadori *et al.*, 1990, Shirai *et al.*, 1992), impaired ability to generate response to recall Ags (Janoff *et al.*, 1991), impaired TD B cell stimulation (Gras *et al.*, 1996, Wolthers *et al.*, 1997) and abnormal cytokine production. Moreover, it has been demonstrated that HIV-infection induces high expression of CD8 T cell antigens (Schlesinger *et al.*, 1996), high expression of Fas ligand protein (Samuelsson *et al.*, 1997) on B cells which enhances the level of apoptosis in these cells. On the other hand, it was reported that HIV-infection causes a low expression of CD40L on TH cells and CD70 on B cells (Wolthers *et al.*, 1997), induces changes in receptor (CD20, HLA-DR, CD21) density (Ginaldi et al., 1998), reduces interleuken-8 receptor (IL-8R) expression on B cells (Jinquan *et al.*, 1997).

1.3.5 T Lymphocyte abnormalities

The normal range of CD4 and CD8 positive T cells count in the circulating blood of adult healthy individuals is found to be between 50-75% and 25-50% respectively, and the balance between these two subsets of T lymphocyte is important in determining the outcome of host immune response. However, HIV infection is known to alter this (CD4+ to CD8+) T lymphocytes ratio (Heinkelein *et al.*,1995, Levy *et al.*; 1996, Rosenberg *et al.*, 1998). Many studies have reported transient elevation in the absolute numbers of cytotoxic T lymphocytes (CTL), after HIV-infection (Ferbas, 1998, Klein *et al.*; 1998, Rosenberg *et al.*; 1998, Rich *et al.*, 1999). This increase in quantity of CD8+ (CTL), cells has been demonstrated to correlate with the fight against a variety of viral antigens, particularly, to epitopes within the Gag, RT, Pol, Env and occasionally to nef, vif, and tat proteins (Rinaldo *et al.*, 1995, Levy *et al.*, 1996). This it is thought to be responsible for the initial reduction of early viremia during primary infection (Poignard *et al.*, 1996, Lubaki *et al.*; 1999, Zou *et al.*, 1999) and the maintenance of asymptomatic phase of infection before the development of AIDS.

The CD8+ T cells, in addition to lysing HIV-infected cells, are presumed to have a suppressive effector function through production of diffusible cytokines that can block virus replication at the level of viral transcription (Levy *et al.*, 1996, Rosok *et al.*; 1997,

Ferbas, 1998, Rich *et al.*, 1999). Moreover, they have been shown to produce β-chemokines (RANTES, MIP-1α and MIP-1β) in response to HIV infection in vitro, and these chemokines are known to block the entry of the virus into the target cells (Ferbas,1998, Garzino-Demo *et al*; 1998).

The progressive loss of CD4+ T cells on the other hand, is well documented as a profound immunological marker which precedes the infection from an asymptomatic phase to AIDS (Chirmule *et al.,* 1995, Heinkelein *et al.;* 1995, Wang *et al.,*1997).

Although the molecular cytopathic mechanisms responsible for CD4+ T cells depletion have not been clearly delineated, a variety of hypotheses that attempt to elucidate the reasons for the CD4+ T cell loss in HIV infection have been proposed. These include direct lysis of the cells by viral infection (Patterson *et al.*, 1995,), syncytium formation (Ryu *et al.*, 1990 Cao *et al.*, 1996), cellular and humoral virus-specific immune responses (Fauci *et al.*, 1991, Zinkernagel and Hengartner,1994), autoimmune reactions (Pantaleo *et al.,*1993), apoptosis (Gougeon and Montagnier, 1993, Maldarlli *et al.*;1995 Wang et al.,1999), antibody-dependent cellular cytotoxicity (Tanneau *et al.*, 1990) and non selective extravasation of CD4+ cells from the blood (Rosenberg *et al.*, 1998). More recently, Borthwick *et al* (1999) have reported that activation-associated necrosis (AAN) greatly contributes to the overall cell death in HIV-infected persons.

The CD4+ subpopulation of the human T lymphocytes known as helper/inducer cells play a central role in the induction of immune responses against a wide range of pathogens as well as malignant tumors. During the induction of an immune response they recognize specific Ag in the context of MHC molecules by virtue of the TCR-αβ heterodimeric–CD3 complex (TCR-CD3). Through the process of Ag recognition, these cells are activated and exhibit responses by releasing different kinds of cytokines which activate the cells of the immune system like B cells, CTL and monocyte/macrophages (Ohno *et al.*, 1991). In addition, these cells secrete factors that affect the growth and differentiation of lymphoid and hematopoietic cells.

Progressive depletion of CD4+ T lymphocytes is well documented as one of the most

and NK cells, but inhibits the proliferation of Th2 lymphocytes. In addition to playing the role of augmenting stimulation of the cellular immune system, it is hypothesized that IFNγ may inhibit HIV-replication (Clerici and Shearer, 1993, Ullum *et al.*; 1997, Bailer *et al.*, 1999). On the other hand, IL-4 which is thought to be produced by Th2, mast cells, basophils and naïve CD4+ T cells (Clerici and Shearer,1993, von der Weid *et al.*, 1996) plays a pivotal role in proliferation and differentiation of naive CD4+ T cells into Th2 cells (Choi and Reiser,1998, Bullens *et al.*, 1999). IL-4 is an obligatory cytokine for IgE production and promotes class switching from IgM to IgG1 and IgE during infection by gastrointestinal parasites (Abbas *et al.*, 1991, Else *et al.*, 1994). IL-5 is known as the major eosinophil differentiation factor (Abbas *et al.*, 1991) while IL-10 is known for inhibiting lymphokine production (e.g IFN-γ) by Th1 cells. Hence, the suggested cytokine production imbalance (switch from a TH1 to Th2 cytokine production pattern) during HIV infection is thought to favour progression to AIDS (Clerici and Shearer, 1993, Olaitan *et al.*; 1998, Bailer *et al.*, 1999).

1.4 **Progression of HIV infection to AIDS**

Immunologic functions are mediated by developmentally independent, but functionally interacting families of lymphocytes. The maintenance of general systemic immunological integrity is dependent on a balanced population of functionally competent Th cells (Heeney *et al.*, 1995). However, many studies have concluded that the hallmark of HIV infection is a profound immunodeficiency resulting primarily from progressive quantitative and qualitative defect in subset of T lymphocytes, known as helper/inducer T lymphocytes (Fauci *et al.*,1988, Meyaard *et al.*; 1993, Coa *et al.*; 1996, Roos *et al.*,1996).

Primary infection with HIV-1 is followed by a burst of viremia and a sudden marked decline in CD4+ T cells. After several weeks, humoral and cellular immune responses are detected. This is followed by the decline of viral load and a rise of CD8+ T cell numbers, while the CD4+ T cell numbers return to a subnormal level. A variable period of clinical latency follows (the average time between infection and AIDS development

ranges from 8 to 12 years). It is usually characterized by a gradual decline of CD4+/CD8+ T cell number, increasing viral load (Fig 2.) and progressive immune dysfunction which finally results in general disturbance of the immune system (Miedema *et al.*, 1988, Poignard *et al.*, 1996).

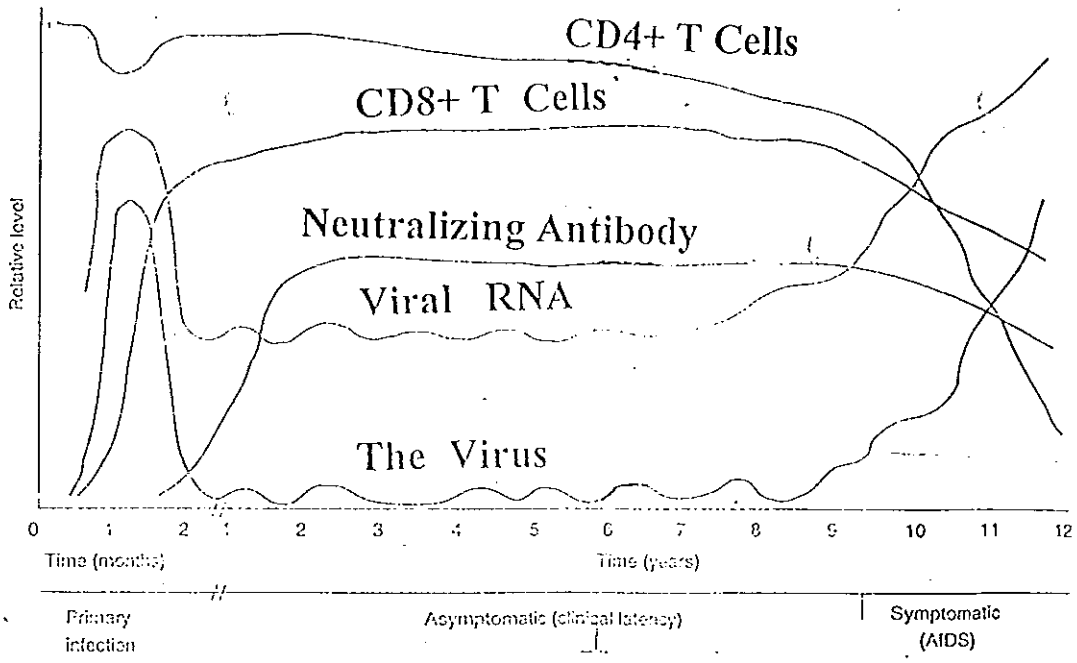


Figure 2. Clinical, Immunological and Virological course of HIV-1 infection progression (Poignord *et al.*,1996).

The asymptomatic phase of infection and the period between AIDS manifestation and death which vary among HIV-infected individuals, seem to be determined by immunologic, viral and host genetic factors in addition to host environmental factors such parasitic infections (Golletti *et al.*, 1996). Parasitic and bacterial infections are known to be inducers of pro-inflammatory cytokines secretion such as tumor necrosis factor-alpha (TNF-α), which can stimulate HIV replication by increasing nuclear factor-

kB (NF-kB) binding to the virus LTR region (Finnegan *et al.,* 1996, Sulkowski *et al.,* 1998). According to Kalinkovich *et al* (1998) helminthic infections enhance immune activation and TH2 profile. These are also considered as the major factors to increase susceptibility and progression of HIV infection in Africa and in other developing countries. It is also suggested that HIV infection progression may be due to changes in biological properties of the virus. In early asymptomatic HIV-1 infection, slow replicating non-syncytium- inducing (NSI) macrophage tropic HIV-1 variants predominate. In the course of infection, the emergence of T-cell tropic (T-tropic) or syncytium inducing (SI) HIV-1 variants may be directly related to the increased rate of CD4+ T cell decline (Abebe *et al.,* 1999), and high viral load and rapid disease progression (Fouchier *et al.*, 1996).

AIDS is the end-stage disease of HIV infection (Weiss, 1993). The clinical transition from the asymptomatic phase to AIDS could involve a profound alterations in number and function of different cells of the immune system (Via *et al.,* 1990, Silveira *et al.*, 1997). The CD4+ lymphocytes count and the plasma levels of the virus have been used as the best measure of the HIV-infection progression. When the CD4+ T cells decline below the level of 200 cells/ul of blood, HIV infected individuals are at high risk of developing a diverse range of opportunistic viral, bacterial, fungal, and protozoa as well as malignancies that describe the established criteria for AIDS. In sub Saharan-Africa, where mycobacterial infection is common, the annual incidence of *Mycobacterium tuberculosis* (TB) is more than 15-fold greater in HIV-infected individuals than in uninfected ones (van de Perre,1995). Studies have suggested that infection with HIV-reactivates the latent TB infection and progresses in to disease. Similarly, infection with TB activates latent stage of HIV infection and hastens infection progression to AIDS and death (De Haas *et al.*, 1998, Toossi *et al.,* 1999). As a result, in Africa TB has become the most common opportunistic infection in HIV-infected individuals.

Before a significant depletion of CD4+ T-cell count and high viral load, various immunological markers have been used to predict HIV infection progression. One of the most studied prognostic markers is the proliferative capacity of peripheral blood

mononuclear cells (PBMC) against common recall antigens and mitogens *in vitro* (Roos *et al.*, 1996, Valentine *et al.*; 1998, Nagy-Agren and Cooney, 1999). Furthermore, a decreased secretion of type 1 cytokines (IL-2, IFN-γ) by T cells isolated from HIV patients following mitogen stimulation of T cells *in vitro* has been documented as a critical step in the progression of HIV disease (Clerici and Shearer, 1993, Clerici *et al.*; 1993, Hyjek *et al.*; 1995, Bailer *et al.*, 1999). However, some authors have hold that a switch from a Th1 to Th2 cytokine phenotype does not occur during the progression of HIV infection (Graziosi *et al.*, 1994, Silverira *et al.*, 1997), whereas others have reported highly increased IFN-γ production by PBMC from asymptomatic HIV infected individuals (Ullum et *al.*, 1997, Nigro *et al.*, 1999). It is also widely proposed that additional environmental factors like infection with helminthes and TB can influence cytokine production and enhance HIV disease progression, especially in most part of Africa (Bentwich et *al.*, 1995, Kalinkovich *et al.*; 1998, Toosi *et al.*, 1999).

In Ethiopia, there are no reliable data that describe the association between prognostic immunological and virological markers (like CD4+ T-cell count, level of plasma RNA of the virus, Th1/Th2 cytokine profile and T-cell proliferative capacity against mitogen phytoheamagglutinin (PHA), and specific Ag like purified protein derivative (PPD) *in vitro*, which are used) to predict HIV infection progression. The study was designed to assess the association between CD4 count, viral load, Th1/Th2 cytokine production (IFN-γ and IL-4) and T-cell proliferative capacity against PHA and PPD in culture of freshly isolated PBMC from both HIV+ and HIV- adult Ethiopian individuals who have been enrolled in the Ethiopian-Netherlands AIDS Research Project (ENARP) cohort study population from Akaki and Wonji Factories. It also is of practical significance to adopt the methodology that is based on the incorporation of the pyrimidine analogue, 5-bromo-2'-deoxyuridine (BrdU) instead of thymidine, into the DNA of proliferating cells to detect T cell proliferation. This is easy, safe and non-radioactive assay used as an alternative method to the most commonly used method of tritiated thymidine incorporation ($[^3H]$-TdR) (Messele *et al.*, 2000).

1.5 The Objectives of the study

General:

- To evaluate in vitro T-cell proliferation as well as cytokine production as marker of HIV infection progression in Ethiopian HIV-infected individuals.

Specific:

- To determine the correlation of in vitro T-cell proliferation by using the BrdU method to CD4+ and CD8+ T- cells count as well as viral load in HIV infection progression.
- • To determine the correlation of in vitro T-cell cytokine production by using the sandwich ELISA method to CD4+ and CD8+ T-cells count as well as viral load in HIV infection progression.
- To investigate the possible association between in vitro T-cell proliferation and cytokine production in HIV infected individuals harboring intestinal parasites.
- To evaluate purified protein derivative (PPD) specific T-cell proliferation and cytokine production in TB/HIV co-infection.

2. Material and Methods

2.1 Study Subjects

A total of 141 age and sex matched (Males and Females, age range between 18 and 48) adult Ethiopian volunteers participating in the EHNRI/ENARP cohort study at the Akaki Fibre and Wonji sugar Factories were enrolled. Of these, 36 subjects were HIV-1-seropositive, while the remaining 105 individuals were seronegative.

2.2 Sample Collection

2.2.1 Blood and Stool Samples

About 10 ml of venous blood drown in coded EDTA vacutainer tubes by ENARP cohort study nurses and physicians at the study sites was transported to the laboratory at ENARP with in 6 hours after collection. After each sample was screened for HIV infection, about 500 ul of whole blood was taken for CD4+/CD8+ T cell count. By the next day Plasma was removed and stored for viral load determination (HIV+ subjects), and serology test for syphilis. The rest of the sample was reconstituted with washing medium and used for PBMC isolation. At the date of blood sample collection, ENARP cohort study staff at the study sites conducted stool analysis for parasitic infection and in vivo tuberculin test, and the result was reported to ENARP.

2.2.2 HIV-screening

Screening for plasma Abs against HIV infection was done by using HIV-SPOT test kits according to the manufacturer's procedure (Genelabs Diagnostics, Singapore Science Park). The HIV-SPOT test determinations were confirmed by enzyme-linked immunosorbent assay (ELISA) and Western blot tests.

a) HIV-SPOT Test

In brief, a drop of plasma sample was added on HIV-SPOT device that was wetted with three drops of reconstituted liquid buffer. Then two drops of liquid buffer and two drops of wash buffer solution were added. This was followed by adding two drops of

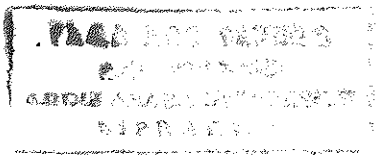
reconstituted conjugate and three drops of washing buffer. Formation of one large distinct red spot in the center of the membrane was interpreted to be positive for Abs against HIV infection. On the other hand, a clear membrane that lacks a red spot at the center of the device was considered to be negative for Abs to the virus.

b) ELISA

Plasma samples were retested for HIV Abs by ELISA using Organo Vironostika kits (Organon Teknika BV, the Netherlands) according to the recommendation of the manufacture. That is 100 ul of specimen diluent was pipetted into 96- ELISA wells plates. 50 ul of serum sample, 3x50 ul of negative control (NC) and 50 ul of positive control (PC) were pipetted into the respective wells. After covering the plate with plastic sealer and mixing well, it was incubated at 37^0C for an hour. Then the wells were washed six times with diluted phosphate buffer and 100 ul of tetramethylbenzidine (TMB) substrate was added into all wells. The plate was re-incubated for 30 minutes at room temperature (RT). The reaction was stopped by adding 100 µl of 1.8 M H_2SO_4. Optical density (OD) was measured at 450nm using an ELISA reader (Reader 230 Organo Teknica Belgium). A sample was considered positive for Abs to HIV, when the measured OD value was greater than the cutoff value and negative when its OD value was less than the determined cutoff value.

c) Western Blot

The Genelabs HIV-1/2 Western blot (Genelabs Diagnostics, Singapore) test was used for further confirmation of the seropositivety of samples previously tested positive by the Organo HIV-1 ELISA. Two ml of diluted wash buffer was added into each well of the Western blot tray. Coded Western blot strips including three controls (strong reactive, weak reactive and non-negative) were placed in the assigned wells and incubated for five minutes at RT. After removing the wash buffer, 2 ml of blotting buffer, 20µl of inactivated sample serum and 20 ul of control serum were added into the respective wells. The tray was covered and incubated over night at RT. By the next day, the wells were washed and 2ml of working conjugate solution (Goat anti-Human IgG diluted



1:1000 in blotting buffer) was added into each well. This was followed by incubation for 30 minutes at RT and adding of 2ml working substrate solution [(5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue teterazolium (NBT)] into each well. The tray was incubated for 15 minutes at RT, and the reaction was stopped by washing four times with distilled water. The presence or absence of Abs to HIV in the tested samples was determined by comparing the bands developed on each nitrocellulose strip to the non-reactive, strong reactive and weak reactive nitrocellulose strips.

2.2.3 CD4+/CD8+ T cell count

Lymphocyte subsets and leukocyte three part differential (granulocyte, monocyte and lymphocyte) were determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) analysis, after staining with commercially available anti- CD3/CD4, CD3/CD8, CD3/CD19 and –CD3/CD16+56 surface molecule monoclonal antibodies (mAb). In brief, 50 ul of whole blood was mixed with 10 ul of each combination of mAbs in four separate tubes and incubated for 15 minutes at RT. Red blood cells were lyzed by incubating with 450 ul of diluted lysing solution (FACSlyse, Becton Dickinson, CA, USA) for 15 minutes at RT. The cells were analyzed using the Cellquest/Multiset software of the FACScan.

2.2.4 Viral load Determination

HIV-1 RNA copy numbers in 100ul plasma samples were quantitated by using a nucleic acid sequence based co-amplification assay (NASBA) using NASBA kits (M.G. and S.J. organon Teknika, N.V., Belgium). In brief, RNA was extracted from 100 ul of plasma stored at –80 ^{0}C using guanidinium isothiocyanatte (GuSCN). That is, plasma samples were lyzed in 5.25M GuSCN, 50 mM Tris/HCl, PH 6.4, 20 mM EDTA and 13% w/v Triton X-100. Nucleic acid was bound by 50 ul activated silica. Silica particles were washed twice with 5.25 M GuSCN, 50 mM Tris/HCl, PH 6.4, twice with 70% ethanol and once with acetone. Nucleic acid was eluted in 50 ul distilled water and aliquoted in 5 ul portions. Ten-fold serial dilutions ranging from 10^2 to 10^6 molecules of Q-RNA were made and mixed with the 5 ul aliquoted nucleic acid isolated from plasma samples. 10 ul

of diluted primers was added to the reaction mixture and then incubated at 65^0 C for 5 minutes to allow primer annealing and subsequently cooled down to 41^0C for 5 minutes. After adding 5 ul of enzyme mixture, amplification was performed in the presence of RNA standards using polymerase chain reaction (PCR). The quantity of amplified RNA was determined by using a NASBA reader (Micro SLT 510, Organon Teknika, Belgium).

2.2.5 PBMC isolation and Count

PBMC were isolated by Ficoll-Hypaque density centrifugation method. In brief, the reconstituted sample with Earel's Balanced salts solution (EBSS) supplemented with 5% new born calf serum (NBCS), 100U/ml penicillin, 100ug/ml streptomycin and 20U/ml heparin was loaded over 12.5 ml ficoll in 50ml test tube, and centrifuged at 14000 rpm for 30 minutes. PBMC were then harvested into a new 50 ml test tube using sterile pasture pipette and washed two times in EBSS medium. The final volume of the pellet was adjusted to 1ml by adding 0.9 ml Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% Fetal Calf Serum (FCS), 100 U/ml penicillin and 100 ug/ml streptomycin to it. From this volume, 20 ul was taken and added into a plastic beaker filled with 10 ml isoton and 3 drops of Triton-100x/saponin. The number of cells/ml were determined by using Coulter counter (Coulter Electronics LTD, U.K.).

2.2.6 T Cell Proliferation Assay

The proliferative response of T cells was determined by using commercial ELISA kits (cell proliferation ELISA system, version 2 Amersham Pharmacia Biotech UK). Briefly, optimal cell concentration was determined by culturing PBMC of $3x10^4$, $5x10^4$, $1x10^5$ and $2x10^5$ cells/well in 200ul of IMDM in triplicate using 96-well flat bottom plate. Cells were incubated for 3 days with 0.5 ug/ml PHA (PHA16 Murex Biotech Limited Central Road, Temple Hill, Dartford, DA1 5LR UK) and 6 days with 10 ug/ml PPD (PPD Batch RT49 for in vitro use, Statens Serum Institute, Denmark, 10 ug/ml) at 37^0C in a 5% CO_2 humidified atmosphere in separate plates. BrdU labeling reagent (final concentration 10 uM) was added to the stimulated and non-stimulated cells and further re-incubated for 24

hours. 72 (PHA) and 144 (PPD) hours later, the cells were centrifuged at 300×g for 10 minutes and the supernatants removed. After drying at 60^{0}C the cells were fixed for 30 minutes in 200ul/well-ethanol solution. Blocking was done by incubating for 30 minutes in 1% w/v protein in (50mM Tris-HCl, 150mM NaCl, pH 7.4,) followed by incubation with peroxidase-labeled anti-BrdU Mab for 90 minutes at RT. Non-bound anti-BrdU antibody was washed with diluted washing buffer. As a substrate, 100ul TMB dissolved in 15% (v/v) dimethyl sulphoxide (DMSO) was dispensed into all wells and incubated for 10 min at RT. Adding 25ul of 1M sulphuric acid into each well stopped the reaction. 120ul of supernatants were collected from each well and transferred into a new 96-well flat bottom plate. OD was measured at 450 nm using an ELISA reader. Proliferative capacity of T cell was calculated as the difference between average of the three replicate stimulated wells and the average of the three un-stimulated control wells.

2.2.7 Cytokine Production Assay

Determination of IL-4 and IFN-γ produced by PBMC from both HIV^+ and HIV^- individuals was performed by culturing PBMC in 24-well plates at a concentration of 10^6 cells/well in 1ml IMDM. The cells were either un-stimulated (negative control) or were stimulated with PHA (0.5 ug/ml) for 3 days, with PPD for 3 and 6 days (10 ug/ml) by incubating at 37^{0}C in a 5% CO_2 atmosphere. At the respective days, supernatants were collected and stored in a freezer at –80^{0}C until assay.

2.2.8 Cytokine measurement

The culture supernatant levels of the IFN-γ and IL-4 were measured by using commercially available ELISA kits (pelikine compact human IFNγ and IL-4 ELISA kits CLB Amsterdam). Briefly, Immuno MaxiSorp plate wells (Nunc-Immuno plate, Denmark) were coated with anti-human Mabs against IFN-γ and IL-4 and incubated overnight at RT. After washing five times the wells of the plates were blocked with blocking reagent by incubating at RT for 1 hour. 100ul of culture supernatant diluted (1:2 for IL-4), (1:10 for PPD) stimulated IFN-γ, (1:100 for PHA) stimulated IFN-γ and serial diluted IFNγ and IL-4 standards in dilution buffer were pipetted in duplicate into all

wells of the respective plates and further incubated for 1 hour at RT. The plates were washed 5 times and dried. 100ul of 1:100 diluted biotinylated IFNγ and IL-4 antibodies were added into the wells except for the substrate blank wells and incubated for 1 hour at RT. Horseradish peroxidase (HRP) conjugated streptavidin diluted (1:1000) in dilution buffer was added and incubated for 30 minutes at RT. The plates were washed, and 100ul of a substrate solution (TMB) was added into all wells and incubated for additional 30 minutes at RT in the dark. Adding of 100ul of 1.8 M H2SO4 solution stopped the reaction. OD was measured at 450nm using an ELISA reader. The concentration of IFNγ and IL-4 were determined in pg/ml against the standards OD values.

2.2.9. Data Analysis

Data were analyzed using stata program. Two-sample t test with equal variances and two-sample Wilcoxon rank-sum test were used to compare two different groups. Differences were considered significant when the P-value was less than 0.05.To evaluate an association between two parameters regression analysis and Spearman's rank correlation test were performed. Association was considered valid when $r \geq = 0.5$, and $p = \leq 0.05$.

3. Results

3.1. T-cell Proliferative Responses and Cytokine production

For *in vitro* T-cell proliferation using BrdU ELISA, the number of cells was optimized by culturing PBMC at different concentrations as shown in Fig 2. The proliferative capacity of T cell was measured by culturing freshly isolated PBMC from both HIV+ (n= 36) and HIV- (n= 105) subjects at a concentration of $1x10^5$ cells/well in triplicate for 3 and 6 days with PHA and PPD respectively.

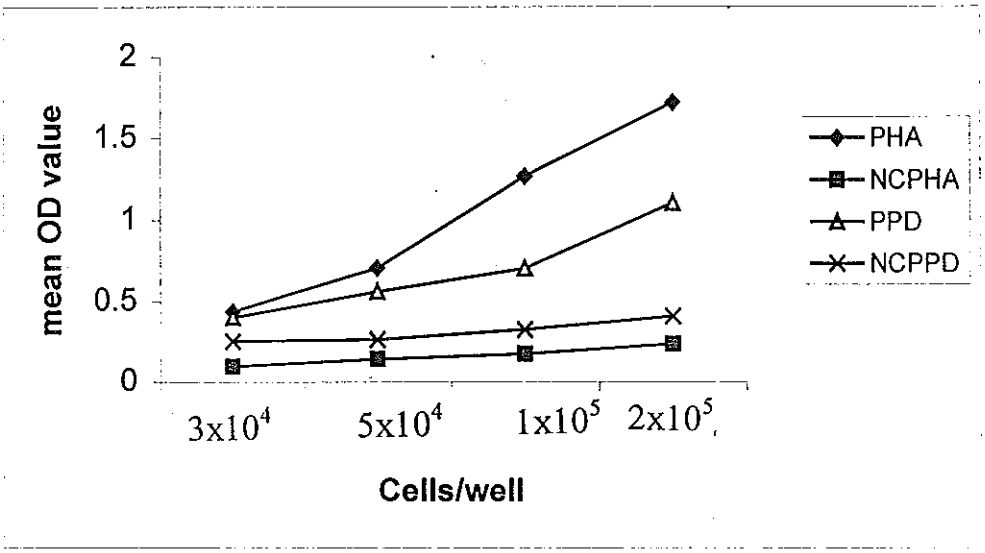
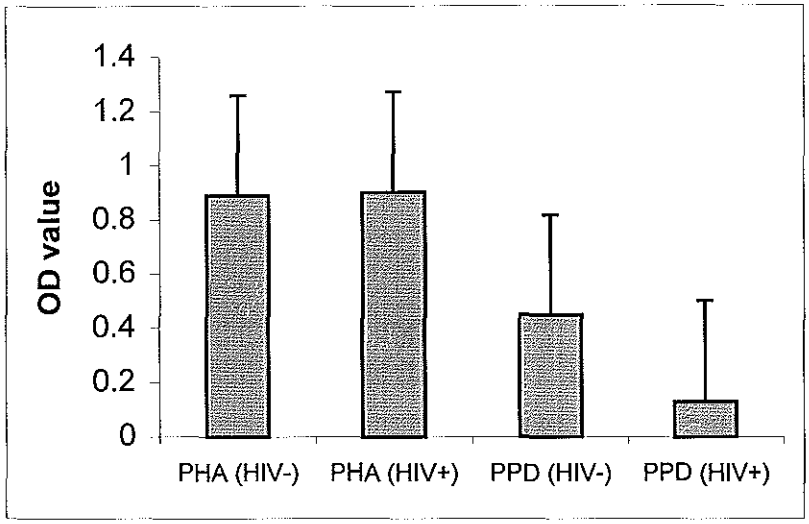


Figure 3. PHA (0.5ug/ml) and PPD (10ug/ml) stimulated PBMC at different cell concentration /well. Results are expressed as mean OD values (n=11). NCPHA= Negative control for PHA; NCPPD = Negative control for PPD.

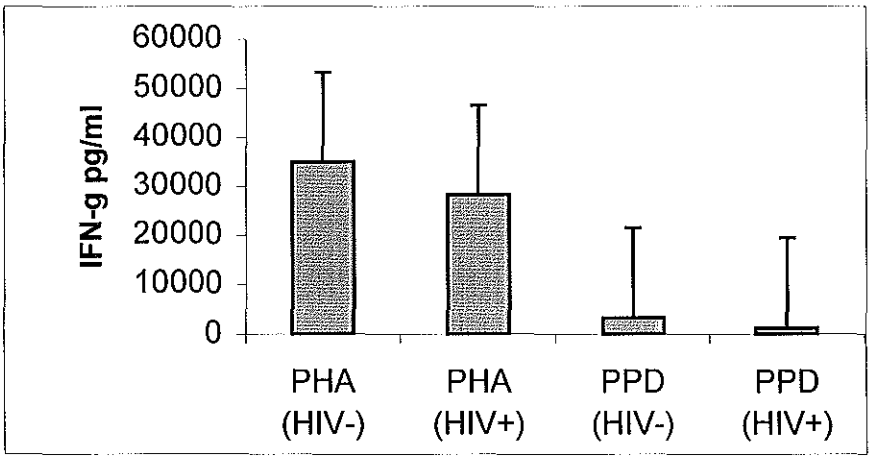
Results of the T-cell proliferative responses and that of cytokine secretion are shown in Fig 4 a & b. There was no difference between HIV infected and non-infected subjects in terms of their T cell proliferative responses to PHA stimulation (p=0.086). In contrast, a significant difference in T-cell proliferative responses to PPD was observed between the two groups (p=0.000).

PPD induced IFN-γ production of PBMC in HIV infected individuals was highly reduced (p = 0.000), whereas that of PHA was only slightly lower than the controls (p=0.061)

(Fig3b). PHA induced IL-4 production by PBMC obtained from both HIV+ and HIV- subjects did not show a difference between the two groups (p=0.566). On the other hand, 3 days or 6 days post PPD stimulation did not induce detectable IL-4 production in both groups.



4 a. proliferative responses



4 b. IFN-γ production.

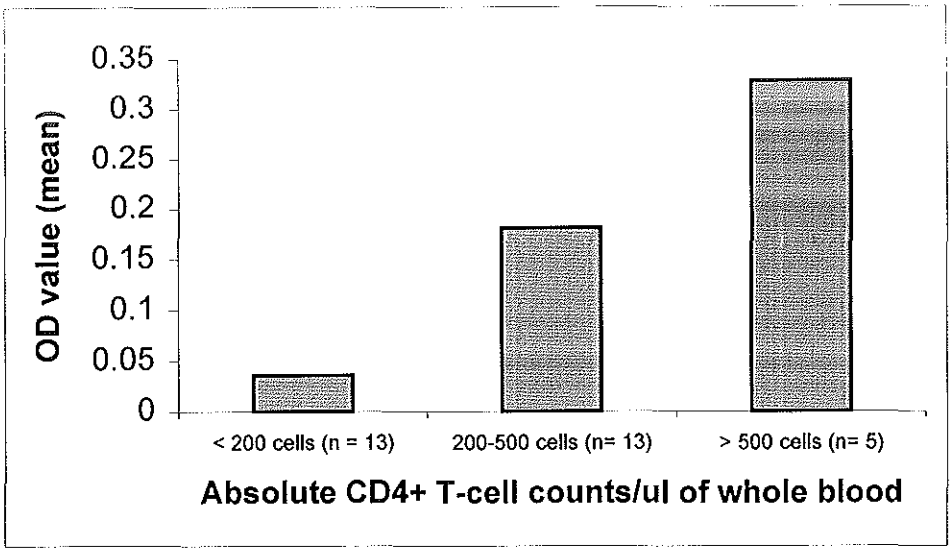
Figure 4. Proliferative response and cytokine production of PBMC to: (4a) PHA; and (4 b) PPD in HIV- (n=105) and HIV+ (n=37) subjects. Results are expressed as mean OD values ± SD.

Comparison of the *in vitro* T-cell proliferative responses to tuberculin PPD was done on 18 HIV+ and 74 HIV- subjects. The results showed a strong positive correlation in HIV- subjects ($r = 0.671$, $p = 0.000$) and no correlation in HIV+ subjects ($r = -0.0284$, $p = 0.9108$). Similarly, in vitro assessment of T-cell proliferative responses to PPD in BCG vaccinated and non vaccinated HIV- subjects showed a significant difference between the two groups ($p = 0.0382$). This difference did not observed in HIV+ subjects ($p = 0.4705$).

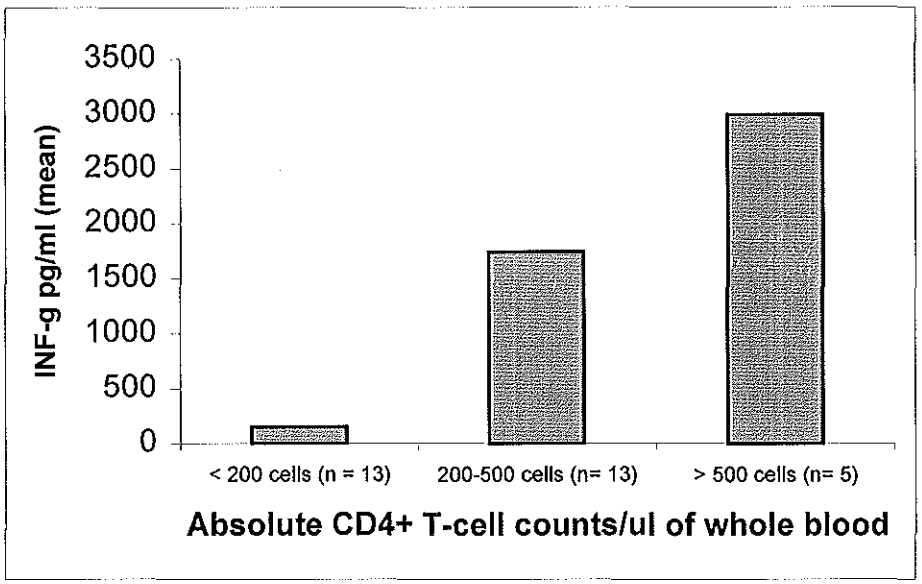
3.2 Association of PHA and PPD induced T cell Responses to CD4/CD8 cells, viral load and intestinal parasite infections in HIV+/ HIV- subjects.

a) Absolute CD4/CD8 counts and CD4:CD8 ratio

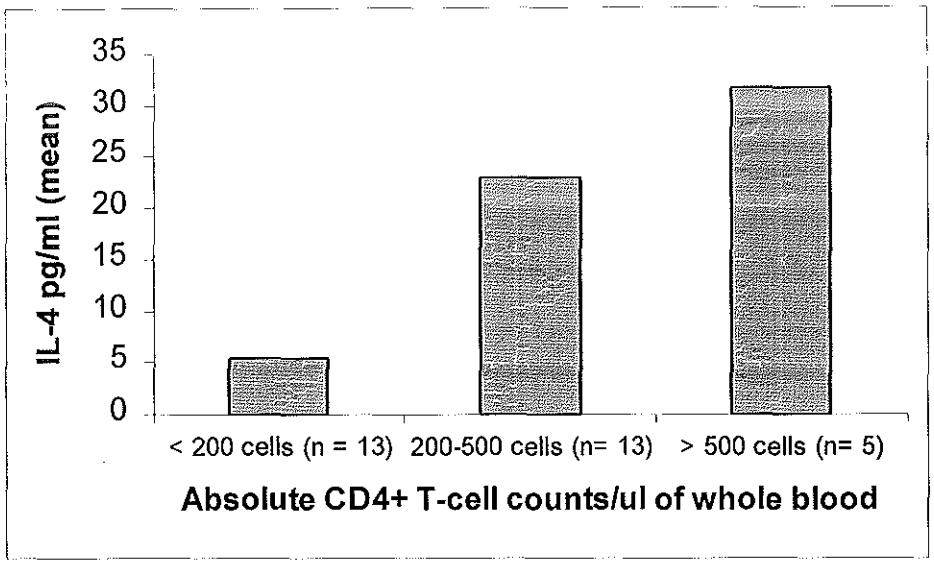
The absolute CD4+ T cell counts of the HIV infected subjects ($n = 31$) ranged between 71-935 cells /ul of whole blood, while that of the control subjects ($n = 64$) ranged between 229-1082 cells/ul. About 42% of the HIV+ individuals had counts below 200 cells/ul. HIV+ subjects were grouped into three groups based on their absolute CD4+ T-cell counts (CD4+ T-cell counts < 200 cells/ul, $> 200 < 500$ cells/ul and > 500 cells/ul of whole blood). Then the mean OD values of T-cell proliferative response to PPD and cytokines (IFN-γ and IL-4) production were compared between the groups. The results showed that there is a significant doifference in mean OD values and cytokine levels between the groups according to their CD4+ T-cell categories ($p < 0.05$) (Fig 5a,b&c). Individuals with more depleted CD4+ T-cell counts (< 200 cells/ul) had a lower proliferative responses to PPD and cytokine production than those who had CD4+ T-cell counts > 200 cells/ul.



5a. CD4+ T-cell proliferative reponses



5b. IFN-γ production



5c. IL-4 production

Figure 5. Proliferative response and cytokine production of T-cell to: (5a &b), PPD; and (5c) PHA in HIV+ subjects based on their CD4+ T-cell count categories.

Comparison of the absolute counts of CD4+/CD8+ T-cell of the HIV+ subjects to the negative ones showed a significant difference between the groups (p=0.000) (Fig 6).

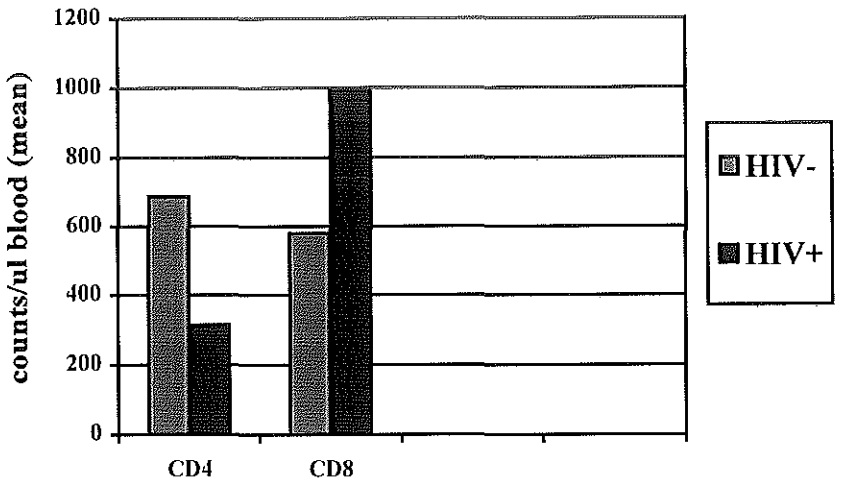


Figure 6. Comparison of absolute CD4+ and CD8+ T-cell counts according to HIV status. Results are expressed as mean of absolute CD4/ CD8 counts/ul. HIV- (n = 64) and (HIV+ (n =31).

Assessment of the relation of absolute CD4 counts and the CD4:CD8 ratio to PPD responses in HIV+ subjects as analyzed by Spearman rank correlation coefficient and regression analysis is shown in Fig 7 a, b, c &d. The absolute count of CD4 as well as CD4:CD8 ratio was positively correlated to the T-cell proliferative responses against PPD ($r = 0.644$, $p = 0.000$). Similarly, PPD induced level of IFN-γ was positively correlated to the CD4 counts ($r = 0.5$, $p = 0.022$). Regardless of high or low CD4 counts, PBMC culture from the same subjects showed similar pattern of proliferative responses towards PHA ($p = 0.863$), whereas PHA induced IL-4 production showed a weak positive correlation with the absolute CD4 counts as well as the CD4:CD8 ratio. Association was not found between the absolute CD8 counts and PBMC responses to PHA and PPD in both HIV+ and HIV- subjects ($p = 0.872$ and 0.172) respectively.

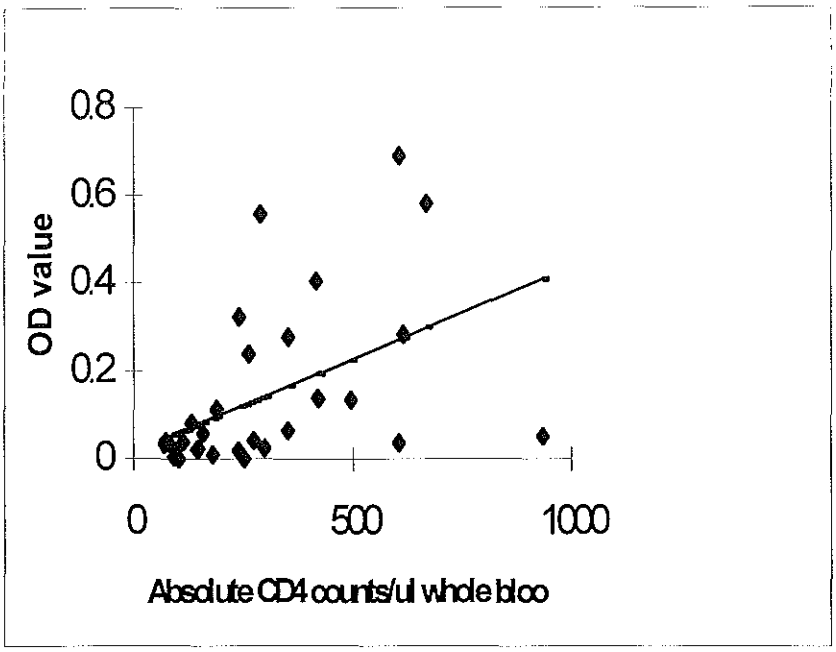


Fig 7a. Association between absolute CD4 counts and PBMC proliferative responses in HIV+ subjects.

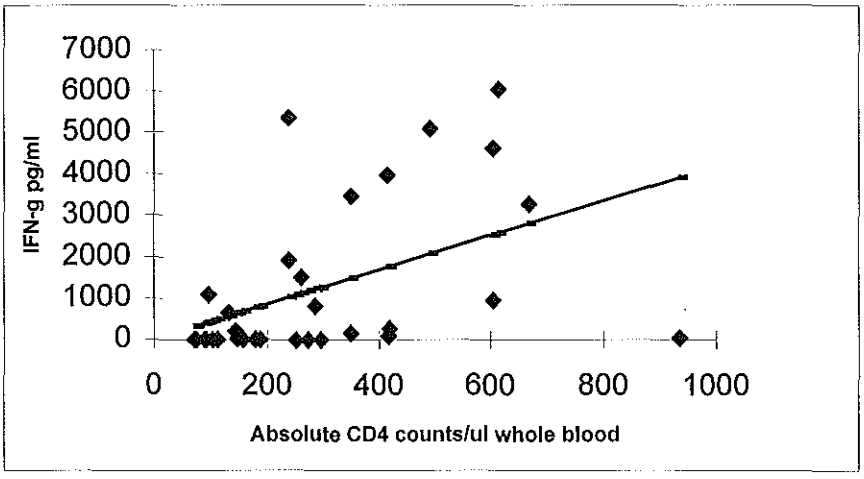


Fig 7b. Association between absolute CD4 counts and IFN-γ production in HIV+ subjects.

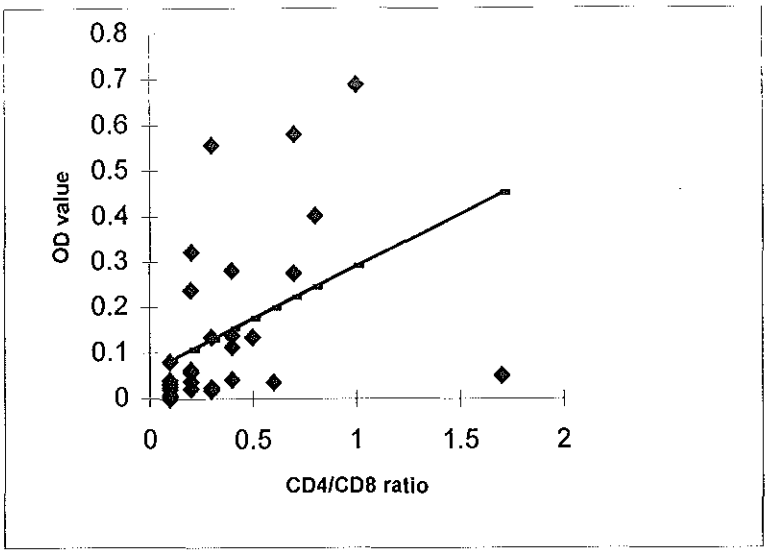


Fig 7c. Association between CD4/CD8 ratio and PBMC proliferative responses to PPD in HIV+ subjects.

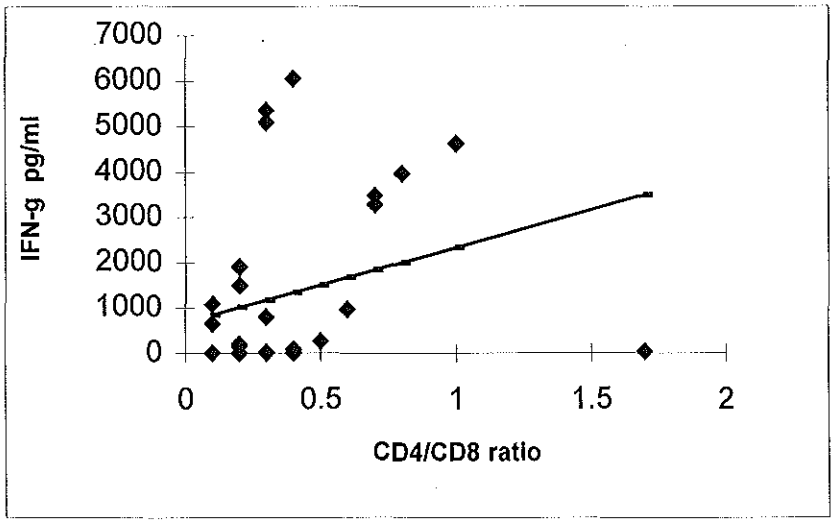


Fig 7d. Association between CD4/CD8 ratio and IFN-γ production by PBMC stimulated with PPD in HIV+ subjects.

b) Plasma RNA viral load

The plasma RNA viral load was inversely correlated to both PPD induced T-cell proliferative responses (r = -0.527, p = 0.001) and IFN-γ production (r = -0.435, p = 0.009) respectively (Fig 10a,b). T-cell responses (proliferation and IFN-γ) of the same individuals did not show such type of significant association against PHA stimulation (r = -0.1, p =0.572). On the other hand an inverse relationship was observed between the level of PHA induced IL-4 and the plasma RNA viral load (r = -0.491, p = 0.003) (Fig 11).

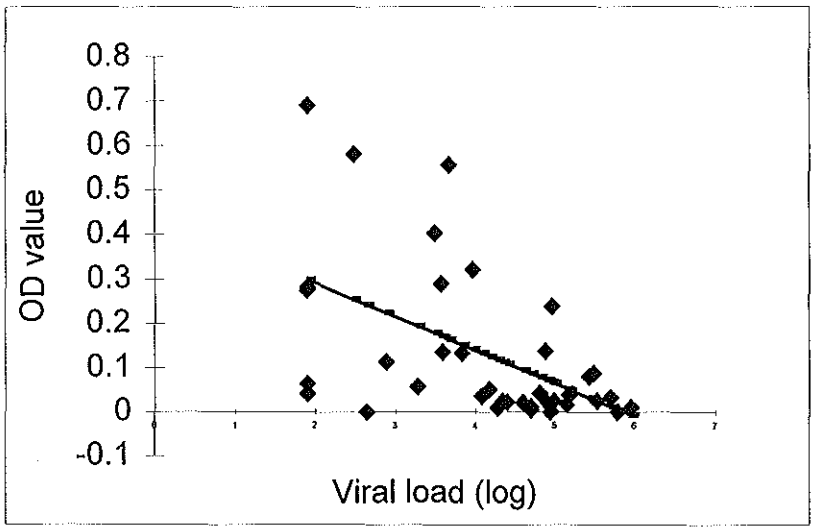


Fig.10 a. Association between plasma RNA viral load and T-cell proliferative responses (n =36).

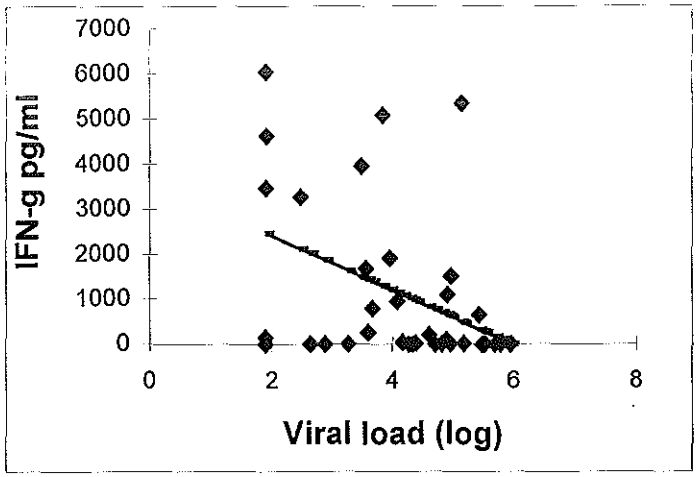


Fig 10b. Association between plasma RNA viral load and IFN-γ production by PPD stimulated PBMC (n = 36).

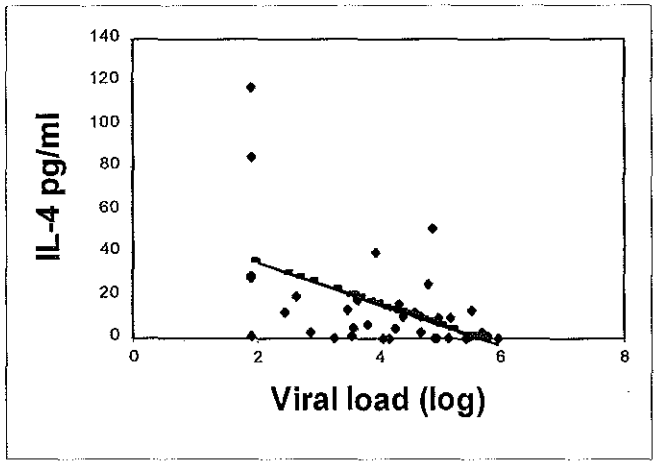


Fig 11. Association between plasma RNA viral load and PHA induced IL-4 production.

c) Parasite infections

Data on the prevalence of parasitic infection in the study population are indicated in (Table1). The relationship of gastro-intestinal parasites and the proliferative response of the cells and cytokine (IFN-γ and IL-4) production was analyzed against protozoan (amoeba and Giardia) and helminth (*Ascaris lumbricoides*, *Trichuris trichura* and *Taenia*

saginata) parasites. The Results showed that although the HIV+ individuals, on the average, showed a higher prevalence of parasite infection, there was no difference in both proliferative and cytokine production between the parasite positive and negative in HIV+ or HIV- subjects (Table 2).

Table 1. The percent prevalence of different intestinal parasites in the study population.

Parasite	HIV-subjects (n=102)	HIV+ subjects (n=37)
Entamoeba histolytica	26/102 (26%)	16/37 (43%)
Giardia lamblia	2/102 (2%)	3/37 (8%)
Ascaris lumbricoides	9/102 (9%)	3/37 (8%)
Trichuris trichuria	3/102 (3%)	0/37 (0%)
Teania saginata	1/102 (1%)	0/37 (0%)
Schistosoma mansoni	1/102 (1%)	0/37 (0%)
Total parasite	43/102 (43%)	22/37 (59%)

Table 2. Comparison of parasite positive and negative individuals (HIV- and HIV+) in terms of PBMC proliferative response and cytokines (IFN-γ and IL-4) production (mean±SD) .

Stimulus	Disease status	proliferation (OD)	cytokineproduction (pg/ml)	
			FN-γ	IL-4
PHA	**HIV- (102)**			
	Parasite (-) n= 60	0.859 ± 0.393*	35271.93 ± 19531	18.57 ± 25
	Parasite (+) n= 42	0.913 ± 0.344*	34886.98 ± 17166	17.25 ± 21
	HIV+ (n= 36)			
	Parasite (-) n= 14	0.766 ± 0.493*	27815.07 ± 18400*	17.79 ± 30*
	Parasite (+) n= 22	0.986 ± 0.379*	28548.91 ± 18776*	13.64 ± 20*
PPD	**HIV- (n=102)**			
	Parasite (-) n = 60	0.457 ± 0.35*	3111.31 ± 2489*	NR
	Parasite (+) n = 42	0.438± 0.373*	3379.98 ± 3933*	NR
	HIV+ (n= 36)			
	Parasite (-) n= 14	0.192 ± 0.245*	1730.29 ± 2113*	NR
	Parasite (+) n =22	0.092 ± 0.117*	771 ± 1541*	NR

NR (no results), * (the difference b/n the two group is not significant).

4. Discussion

Assessment of prognostic immunological and virological markers like CD4 cell counts, levels of plasma RNA or cell-associated viral load, *in vitro* T lymphocyte proliferative capacity to mitogen and recall antigen, and the pattern of cytokine production that possibly appear as the outcome of infection with HIV have been used to predict the pathogenesis of HIV-infection and infection progression in many developed countries. These require well equipped laboratories and the use of radioactive materials for the detection of the T-cell proliferative response. To fill this gap, relatively simple alternative methods: the BrdU ELISA for use in the assessment of T-cell proliferation and the sandwich ELISA for cytokine study were employed.

The *in vitro* T-cell proliferation was optimized in terms of the number of cells required for the test system and other conditions that minimize the background. Using this optimized method, a total of 142 subjects of which 36 were HIV-1 seropositives and the rest 105 HIV-1 seronegative controls were assessed. The HIV-1 infected individuals were clinically indistinguishable from the non-infected controls. PBMC obtained from these groups did not show a difference in response to the general mitogen, PHA when compared to the negative controls. In contrast, a proliferative responses to the recall antigen PPD was found to be significantly lower in HIV+ subjects, compared to HIV- controls. These results are in conformity with previous works (Hofmann *et al.*, 1989, Clerici and Shearer, 1993, Meyaard *et al.*; 1993, Nagy-Agren and Cooney, 1999). The reason for maintenance of normal T-cell responses to mitogens like PHA and loss of it to recall antigens during early HIV infection is not well documented. As Hofmann *et al.* (1989), however, described it, PHA stimulates T-lymphocytes through CD2 receptors which unlike CD3 is not affected during the early stage of infection.

Microbial antigens like PPD upon presentation to T-lymphocytes induce proliferation signals through the interaction of TCR-heterodimer-CD3 complex, MHC-class II and CD4 molecules (Ohno et al., 1991). Strong lymphocyte proliferative responses to microbial antigens measured in vitro assess indicates the presence of normal immunologic response that also occurs in vivo (Valentine et al., 1998). Delayed-type

hypersensitivity (DTH) skin testing and measuring in vitro T-cell responses against the same recall antigens like PPD, provide information about the association between in vitro and in vivo immunologic responses to that antigen. In addition, DTH skin testing has been shown to predict HIV infection progression to AIDS as well as for clinical staging (Dolan *et al.,* 1995). Considering the fact that the subjects in this study were some vaccinated with Bacillus Calmette Guerin (BCG) one would expect them to have memory cells to PPD both in vivo and in vitro. A significant difference was observed between BCG vaccinated and non vaccinated HIV- subjects in terms of proliferative responses to PPD in vitro ($p = 0.0382$). Moreover, a strong association was found between the in vitro and the in vivo PPD responses in HIV- individuals. On the other hand, difference was not observed between proliferative responses of PBMC obtained from BCG vaccinated and non vaccinated HIV+ individuals. This shows that HIV infection affects the immune response to this recall antigen as documented earlier (Valentine *et al.*, 1998). In this regard, many mechanisms that are likely to be involved in the immune dysfunction are suggested: Chirmule *et al.*(1995) suggested binding of HIV-gp120 to the CD4 surface molecules to be interrupting the sequential cascade of intercellular interactions; Chun *et al.* (1997) proposed selective HIV-induced depletion of memory T-lymphocytes, while Connors *et al* (1997) reported loss of receptor specificity of CD4+ T cells to recall antigen. The possible interference of HIV in antigen presenting functions of accessory cells is also for worded by Meyaard *et al* (1993).

It is known that the immune system as a strategy of protection uses T-cell subsets that are defined by their cytokine pattern: Th1 responses produce (IFN-γ and IL-2) which are characteristically associated with resistance and elimination of intracellular pathogens such as *Mycobacterium tuberculosis* and different viral infections. Th2 responses like (IL-4, IL-5 and IL-10), on the other hand, are elicited by extra-cellular parasite infections and correlate with protective immunity (Pearlman *et al.*, 1993).

Two distinct explanations are proposed for pattern of cytokine production during HIV-infection. Some have suggested that HIV infection could result in enhanced Th2 responses (IL-4 and IL-10) which down regulate Th1 responses (IFN-γ and IL-2). And

this imbalance of cytokine secretion plays a role in the progression of HIV infection to AIDS (Clerici and Shearer, 1993, Clerici *et al.*, 1993). Contrary to this two independent studies by Graziosi *et al* (1994) and Silverira *et al* (1997) have shown that such a shift does not exist in HIV infection progression.

Taking this into account, the cytokine secretion pattern of T-cells corresponding to PHA and PPD stimulation was assessed. PHA stimulation induced high production of IFN-γ and relatively detectable IL-4 from PBMC of both HIV+ and HIV- subjects. PPD stimulation on the other hand, in both groups (HIV+ and HIV-), failed to produce IL-4 in response to it. This observation was reported previously by De Prete *et al* (1991) and Sartono *et al* (1996) that PPD stimulated PBMC cultures produced high levels of IFN-γ but failed to produce IL-4.

PPD being an antigen from intracellular parasite was expected to induce Th1 type response for protection. Since some (HIV+ and HIV-) study subjects have had exposure to BCG at one time or another, they would be presumed to have developed a Th1 memory cells in their immune system. However, we detected a highly reduced PPD induced IFN-γ production in the culture supernatants of PBMC isolated from HIV infected asymptomatic subjects. This is consistent with the investigation by Harrison and Levitz (1997). They showed a reduced IFN-γ production by PBMC from HIV-seropositive subjects in response to other recall Ags like *Crytococcus neoformans, Candida albicans and Mycobacterium tuberculosis.*

Clerici *et al* (1993) and Clerici and Shearer (1993) from their observations of IL-2 and IL-4 secretion of asymptomatic HIV-infected subjects' PBMC over time, they proposed a switch from the Th1 to Th2 cytokine phenotype. In our study, comparison of the level of PHA induced IL-4 in the culture supernatants of PBMC from both HIV+ and HIV- control subjects did not show a difference in IL-4 production between the two groups ($p = 0.415$). It seems as Bailer *et al* (1999) observed during their early phase of infection HIV+ subjects show a reduced response to recall antigens with a dominant type 1 cytokine secretion rather than a shift to Th2 type response.

It is widely accepted that as the primary target of HIV infection, CD4+ T cells are preferentially infected and depleted with progression of the infection. Thus, for a person infected with HIV, CD4 counts has been used as one of the surrogate marker to provide important information about the immune status of that individual as well as to predict the risk of AIDS development (Phillips *et al.*, 1991, Morgan *et al.*, 1999). Jobe *et al* (1999) reported that HIV-induced immuno-suppression is related to depletion of CD4+ T cells within peripheral blood. Therefore, in order to investigate the above observation in our study population by the BrdU METHOD, we first analyzed CD4 and CD8 absolute counts in both HIV+ and HIV- study subjects. The results showed that HIV-infected individuals had a low CD4 but high CD8 counts, whereas HIV-uninfected control subjects had relatively high CD4 and low CD8 counts. This observation is in consistent with the works of others that following primary HIV infection, most individuals experiences a significant alteration in the ratios of circulating T lymphocyte subsets due to CD4+ T cell depletion and CD8+ T cell expansion (Levy *et al.*, 1996, Chun *et al.;* 1997, Rosenberg *et al.*; 1998, Tedla *et al.,* 1999, Messele *et al.1999*). Examination of the association between absolute CD4 counts and T-cell proliferative responses to PPD and PHA revealed that the reduced T-cell responses (proliferation and IFN-γ secretion) to PPD were positively correlated with the absolute CD4 cell counts in HIV+ subjects (Fig 5a & b) but not in the case of the controls. These observations are in agreement with the findings of Ravn *et a*l (1997) who reported CD4+ lymphocytes to be the main source of IFN-γ during *in vitro* stimulation with soluble mycobacterial antigens and stands against the suggestion that CD8+ T cells are the source of high IFN-γ production during asymptomatic phase of HIV-infection (Ullum *et al.*, 1997).

The above observation is also reflected on the pattern of CD4:CD8 ratio. Evaluation of the association between CD4:CD8 ratio and PBMC proliferative responses indicated that both proliferation and cytokine (IFN-γ) secretion can be affected by the status of CD4:CD8 ratio of the individuals. Those subjects who had relatively low CD4:CD8 ratio had lower PBMC proliferative response to PPD and IFN-γ secretion than those who had high CD4:CD8 ratio (Fig 7c,d).

Comparatively higher level of PPD induced IFN-γ was observed in HIV infected study subjects who had relatively high CD4 counts. The level of PHA induced IL-4 in HIV+ individuals reflected a similar association as that observed between IFN-γ production and CD4 counts. Similar to observation by Maggi *et al* (1994) these results indicated that PBMC obtained from HIV-infected subjects with a reduced CD4+ T cell count produce lower amounts of both cytokines (IFN-γ and IL-4), in comparison to those individuals with relatively less decrease in CD4+ T cells counts. This is not in agreement with the observation that a reduced IL-4 production in HIV infected subjects with high CD4 counts (Hyjek *et al.,* 1995, Meroni *et al.:* 1996, Sousa and Victorino,1998).

In sub-Saharan Africa the association between the plasma RNA levels of the virus and the T-cell responses has not been well documented. Therefore, in order to assess the outcome of high plasma RNA viral load on T cell proliferative capacity and cytokine production, we measured plasma RNA viral load and compared these results with proliferative responses and cytokine (IFN-γ) production induced by PPD. The results showed that PBMC obtained from individuals whose plasma contains low RNA viral load had relatively better T-cell responses to PPD (Fig10a&b). These observations confirm the findings that cytokine secretion levels correlate positively with CD4 cell counts and negatively with plasma RNA HIV-1 viral load (Bailer *et al.*, 1999, Jobe *et al.,* 1999).

In general chronic human infections, the specific immune response to the pathogen may be of vital importance to host defense. It is this immunologic response that contributes to resistance or susceptibility to different infectious agents. Resistance seems to depend upon the presence of functionally distinct CD4+ T cells. Th1 cells which release (IFN-γ and IL-2), appear to participate in protective immune responses, whereas Th2 cells, which release cytokines like IL-4, IL-5 and IL-10, are more often associated with pathology (De Kossodo and Grau *et al.*, 1993). However, the host immune system has its own immune balance control mechanisms. And that Th1 and Th2 responses can develop independently within the same individual against different infectious agents at the same time (Sartono *et al.,* 1996). Studies have suggested that parasitic infection especially

intestinal parasites can modulate Th1 responses toward the Th2 responses (Kalinkovich *et al.*, 1998, Messele *et al.*, 1999) and contribute to a decreased activity of Th1 responses (Pearlman *et al.*, 1993). Moreover, Bentwich *et al* (1995) have suggested that HIV infected individuals co-infected by helminthes progress more rapidly to AIDS stage than infected by HIV only. Thus, in areas like Africa where there is a high burden of parasite infection, a Th1 to Th2 switch due to parasitic infection could contribute to HIV infection progression.

In this study, the effects of intestinal parasitic infection like amoeba, Giardia and Ascaris on T cell proliferative responses and cytokine production (IFN-γ and IL-4) against PPD and PHA stimulation was evaluated in both HIV infected and uninfected Ethiopians. Despite the presence of these intestinal parasites in the study population (HIV+ 60% and HIV- 43%), no association could be found with the T cell proliferative responses, IFN-γ secretion and/or an increased IL-4 production. These observations do not support the notion that the functional impairment of T cells such as reduced proliferation and type 1 cytokine production to recall antigen and mitogen, to be due to parasitic infection (Bentwich et al., 1998, Daniel, 1999). We therefore, are of the opinion that the functional abnormality of T cells (reduced proliferation and IFN-γ secretion) observed in HIV-1 infected study subjects is due to the activity of the virus rather than that of the parasites.

The results indicated the fact that infection with HIV induces an impaired T–cell proliferative responses and diminished type 1 cytokine (IFN-γ) production. These T-cell responses (proliferation and cytokine secretion) can be assessed by using a relatively simple alternative methods: BrdU ELISA for the proliferation assay and the sandwich type of ELISA for the cytokine detection after *in vitro* stimulation of PBMC with common recall antigens like purified protein derivative of *Mycobacterium tuberculosis*. And, this evaluation of immunological dysfunctions in early HIV infection against common recall antigens might have important implication for vaccine design and trials, to predict infection progression as well as to protect late stage of HIV-infected individuals from infection with various opportunistic infections.

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